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INVITED REVIEW

Manipulating megakaryocytes to manufacture platelets *ex vivo*

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Summary. Historically, platelet transfusion has proven a reliable way to treat patients suffering from thrombocytopenia or similar ailments. An undersupply of donors, however, has demanded alternative platelet sources. Scientists have therefore sought to recapitulate the biological events that convert hematopoietic stem cells into platelets in the laboratory. Such platelets have shown good function and potential for treatment. Yet the number manufactured *ex vivo* falls well short of clinical application. Part of the reason is the remarkable gaps in our understanding of the molecular mechanisms driving platelet formation. Using several stem cell sources, scientists have progressively clarified the chemical signaling and physical microenvironment that optimize *ex vivo* platelets and reconstituted them in synthetic environments. Key advances in cell reprogramming and the ability to propagate self-renewal have extended the lifetime of megakaryocytes to increase the pool of platelet progenitors.

Keywords: bioreactors; blood platelets; induced pluripotent stem cells; megakaryocytes; polyploidy.

Introduction

Platelets are best known for their role in wound repair, but have responsibility in several other functions, including innate immunity, vascular integrity, and neoangiogenesis. The human body has several hundred billion circulating platelets at any one time and recycles its entire platelet population every 10 days [1]. Because of its long history and success, platelet transfusion has remained the most popular way to provide platelets to patients suffering from thrombocytopenia and other ailments that require an external platelet source. However, because platelets must be preserved at room temperature, they risk bacterial contamination, which gives them a shelf life of only a few days. Thus, donors are continuously being sought to replenish a fragile supply that often sees unacceptable proportions go to waste [2]. Improved life spans and medical technologies have only intensified this demand at a rate which platelet donors have not kept up. Indeed, in some nations, it has been estimated that the donor population will underserve by 20% [3]. Thus, alternative sources for platelets have received attention, especially those that generate platelets *ex vivo*.

One example is hematopoietic stem cells (HSCs), which can be differentiated to megakaryocytes (MKs), the unipotent progenitor of all platelets, and acquired from umbilical cord blood (UCB). A typical platelet unit for transfusion will contain in the neighborhood of 500 billion platelets, and the average MK in the body produces 2000–5000 platelets, but a typical UCB unit only 1 million HSCs [4]. Therefore, assuming 1000 platelets can be gathered from a single MK in the laboratory, HSCs will have to be expanded at least 100 times. However, current *ex vivo* techniques generate fewer than 100 platelets per MK, meaning the expansion will have to be even greater [5].

To achieve these numbers, scientists have comprehensively investigated the chemical signaling and physical microenvironment that promote MK differentiation, maturation, and the release of platelets into the blood stream. Accordingly, a number of groups have designed bioreactors that recapitulate the microenvironment to promote these events. Complementing this strategy are cell reprogramming methods that take advantage of the limitless proliferation of stem cells to generate self-renewing MKs. In this review, we examine the biological steps considered essential to platelet generation and give attention to methods that promise to acquire sufficient *ex vivo* platelets for clinical application.

From megakaryocyte to platelet

Megakaryocytes

Platelets are the anucleated fragments of MKs. In the most accepted hierarchical model, HSCs, or $CD34^+$ cells,

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take the MK lineage through a number of intermediates, with MK-erythroid precursor (MEP) being the penultimate stage [6]. Upon maturation, MKs extend proplatelets, which traverse into the sinusoidal vessels of the bone marrow where they are shred by blood flow into platelets [7]. By this point, the cells will have switched their distinctive markings from $CD34^+$ to $CD41a^+CD42b^+$. Strategies for platelet generation *ex vivo* use this model as the paradigm. Yet as a testament on how much there is still to learn about thrombopoiesis, two recent studies have found that HSCs show surface markings that bias their fate to the MK lineage well before the MEP stage [8,9].

During the differentiation process, a HSC will enter the osteoblast niche and migrate to the perivascular niche to achieve full differentiation. Three transcription factors, GATA1, RUNX1, and NF-E2, are considered the primary determinants of whether MEP will take the MK lineage and proceed with this migration [10]. Thrombopoietin (TPO) is the primary cytokine responsible for the differentiation and binds to c-MPL receptors on $CD34^+$ cells to lead them to the osteoblast niche [11]. The discovery of TPO is considered a cornerstone to our understanding of platelet generation. Indeed, a year after this discovery, the first report to describe *in vitro* platelet generation was published [12]. The elimination of either TPO or c-MPL receptors results in severe thrombocytopenia and reduces the number of MK progenitors and mature MKs [13]. Once in the osteoblast niche, $CD34^+$ cells interact with collagen I via GPVI and $\alpha_2\beta_1$ [14]. The stability of this niche depends on the protein-tyrosine phosphatases Shp1 and Shp2, which regulate the expression of GPVI and Mpl, respectively [15]. In addition to TPO, several studies have demonstrated that a minimal concoction for $CD34^+$ expansion includes stem cell factor (SCF) and at least one other cytokine [5]. Notch signaling via activation by the Delta-1 ligand has been reported to increase expansion 100 times *in vitro*, and Stem Regenin 1, a purine derivative, has been found to have a significant positive effect on human $CD34^+$ cells by blocking aryl hydrocarbon receptors [16,17]. More recent studies have found small molecules can have positive effects on $CD34^+$ cell expansion using other mechanisms of action [18,19].

The maturation of MKs is marked by a massive increase in size, as these cells can reach up to 100 μm in diameter. The reason for the large size is that MKs switch from mitosis to endomitosis due to a failure in cytokinesis, which is due to an abnormal contractile ring caused by the absence of myosin II and regulation by RUNX1 [20,21]. While endomitosis explains how fully mature MKs can reach DNA content up to 128N, MKs have been observed to mature using either mitosis or endomitosis, which would explain the variable ploidy in blood and why less ploidy is sufficient for shedding platelets [22]. This increase in size leads to the invaginated membrane system (IMS), which acts as a membrane reservoir for proplatelets. Proplatelets enter the sinusoidal vessels of the bone marrow and are shred into platelets by blood flow at the very last stage of thrombopoiesis. Polyploidy is thought important for MKs to accumulate the cytoplasmic content that will eventually be used for platelet formation. Indeed, it has been argued that ploidy level correlates directly with the number of platelets [23]. Exceptions to this claim are known, however [24,25].

The regulation of the mitosis–endomitosis transition appears to involve multiple mechanisms. Gao *et al.* [26] showed that GEF-H1 and ECT2 must be downregulated for endomitosis, but that their downregulation occurs sequentially: GEF-H1 occurs in the 2N–4N transition, whereas ECT2 occurs thereafter. Whether these molecules make useful targets for increasing the ploidy of MKs in *ex vivo* platelets generation has not been explored.

As endomitosis proceeds, so too does the size of the IMS, which will eventually disperse throughout the MK. The forces required for the invaginations are initiated by phosphatidylinositol 4,5-bisphosphate, which activates the WASP–WAVE pathway, which in turn promotes actin assembly [27,28]. Interestingly, despite the ubiquity of the IMS in MKs, its beginnings are localized at one region of the surface membrane, which is marked by GPIb receptors [29]. Not coincidently, GPIb receptors are also markers for MK maturation and the transition to the perivascular niche by the cytokine SDF1 and its receptor CXCR4, which have been observed to accelerate the polyploidization of MKs [30,31]. The perivascular niche is made up of several extracellular matrix proteins including von Willebrand factor (vWF), fibrinogen, and fibronectin and is where MKs will begin to extend proplatelets using the organelles and granules accumulated by endomitosis. GPIb-IX-V is the receptor for vWF, a glycoprotein that facilitates platelet adhesion to the subendothelium. vWF appears to have an important role at the very end stages of platelet generation, as its absence has been associated with fewer platelets being shed from MKs [32,33]. Fibrinogen binds to $\alpha_{IIb}\beta_3$ upon MK maturation and can be used to promote proplatelets [34,35]. Finally, fibronectin promotes proplatelet formation by binding to the receptors VLA-4 and VLA-5 [36].

Proplatelets

Visually, proplatelets have numerous swellings that give them the shape of a chain of dumbbells, with each swelling containing the components necessary for a functional platelet. Once in the bloodstream, the proplatelet will be eviscerated at the center of these dumbbells, resulting in two approximately symmetric daughters that can continue to be eviscerated until platelet-sized entities emerge [37]. A single MK will continue to extend proplatelets until finally no more of its cell body remains and its remnants are degraded. It has even been observed that platelets them-

selves extend protrusions that can be further shed into structures that show surface markings indicative of normal platelets [38]. How the system knows when to end this reductionism is not clear.

Proplatelets are dynamic structures continuously changing their shape [37]. Cytoskeletal proteins are constantly transporting organelles and granules bidirectionally to the proplatelets, which are regularly branching to extend more surface area that will increase the number of platelets [39,40]. However, these dynamics are nothing but inefficient if they do not guide the proplatelet to the sinusoidal blood vessels. Using mutant mice, Zhang *et al.* [41] showed the important role of sphingosine 1 phosphate (S1P) and its receptor, S1pr1, on proplatelet formation and platelet shedding. Knockdown of S1pr1 led to a significant decrease in platelet count. Furthermore, the mechanisms leading to the last stages of platelet formation appeared unperturbed, as the number of MKs was unchanged, the establishment of the IMS was normal, and MKs retained their strong proximity to the sinusoidal vessels. Rather, what did appear disrupted was the proplatelet preference for the vessels, as the proplatelets protruded in random directions suggesting insensitivity to the S1P gradient between the lumen and blood.

Even if proplatelets are lured to the endothelial wall by the S1P gradient, they must still find a way to penetrate and enter the blood stream. Schachtner *et al.* [42] found that podosomes on the MK surface can degrade the extracellular matrix, which may provide the gateway for proplatelets to enter the vessels. Podosome activation depends on WASP signaling, as podosomes are actin-rich structures, which is consistent with the degradation being reduced by blebbistatin, a myosin II inhibitor. Interestingly, blebbistatin has been shown to promote platelet generation *ex vivo* [43,44].

Generating platelets in the laboratory

Immortalized MKs

Using the knowledge gained about the differentiation of MKs and the shedding of platelets, scientists have sought to induce $CD34^+$ cells to the MK lineage. $CD34^+$ cells can be acquired from several sources, including bone marrow, peripheral blood, or UCB. MKs from UCB are generally smaller in size and ploidy [45]. In addition, they shed significantly fewer platelets and show distinctive characteristics from MKs in adult blood [23,46]. Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are immortal and in theory can provide a limitless number of $CD34^+$ cells. Bluteau *et al.* [47] showed by global transcriptome analysis the transcription factor network and signaling pathways of megakaryopoiesis in hESCs are more similar to those in HSCs from neonatal blood than those from adult blood, suggesting studies using UCB can be extrapolated to hESCs and hiPSCs.

Gaur *et al.* [48] were the first to show how human hESCs can be differentiated into MKs by culturing them with OP-9 stromal cells. However, these MKs did not exceed a ploidy of 32N and the number generated per starting hESC was less than one. Lu *et al.* [49] were able to generate functional platelets that bound to fibrinogen and vWF starting with hESCs cultured in TPO, SCF, and IL-11. Several hESC lines were expanded approximately 100-fold into MKs, but only 15% of these MKs were $CD41a^+CD42b^+$. Vascular endothelial growth factor (VEGF) may be another molecule that facilitates expansion, as it was found to generate sac-like structures that seemed to harbor environments more suitable for obtaining a number of hematopoietic progenitors, including MK progenitors, and also maturing MKs that shed functional platelets [50]. Here too, however, the number of platelets is untenable for clinical purposes. Additionally, the platelets observed in these *ex vivo* studies were larger and more heterogeneous in size than those found in blood. One possible explanation is that the *ex vivo* generation was carried out in static culture, which did not mimic blood flow eviscerating proplatelets to proper size.

Because they appear to function equivalently, hiPSCs offer a preferred option to hESCs for several reasons, including the absence of ethical controversies and the ability to make any somatic cell in the body a potential source for MKs. One of the more important developments in thrombopoiesis using hiPSCs has been the reporting of immortalized MKs. These cells possess unlimited replication potential while still retaining unipotency, which mitigates the concern of contaminating cells. Nakamura *et al.* [51] have reported a way to extend the lifetime of hiPSC-derived MKs from 2 months to 5 months, a period that could potentially compensate for the low number of platelets shed by MKs *ex vivo*. Key to this accomplishment was the manipulation of c-Myc, a transcription factor stimulated by TPO and one of the four Yamanaka factors used to induce pluripotency in somatic cells [52,53]. Overexpression of c-Myc has been found to increase the proliferation of MKs in mice, but at the cost of decreased maturation and polyploidy [54]. Taking advantage of the aforementioned sac-like structures, Takayama *et al.* [55] found that c-Myc expression was accompanied by an increase in the expressions of p14 (ARF) and p16 (INK4A). Like c-Myc, the activation of these two apoptotic factors has been associated with an inability of MKs to mature and increase their ploidy, perhaps explaining the dysfunctional platelets seen by Nakamura *et al.* and the need to control the expression of these three factors temporally [56,57]. To resolve this problem, Nakamura *et al.* used a destabilization domain vector system to more precisely regulate c-Myc expression and incorporated BMI1, a polycomb complex component, to do the same with ARF and INK4A expression [58,59].

However, even then, MKs showed aberrant properties including normal $CD41a^+$ levels but reduced $CD42b^+$ levels due to the activation of caspases. This problem was resolved by adding BCL-XL [60], an anti-apoptosis agent that has been associated with platelet shedding, to the culture. Important was to add BCL-XL sequentially, two to three weeks after introducing c-Myc and BMI1, as mixing all simultaneously had no beneficial effect. The reduced expression of these factors was accompanied by an increase in the expressions of GATA1 and NF-E2. That c-Myc and other factors must be controlled in a relatively small time window suggests the signaling program for platelet generation is an ongoing dynamic process that can be easily perturbed. As an example of this sensitivity, another study has shown that c-Myc and BCL-XL can be used to tip MEP to erythrocyte lineage [61].

However, like others before it, immortalized MKs do not provide the requisite number of platelets, as each only sheds approximately 3–10 platelets [51]. Moreover, the platelets showed less robust response than fresh human platelets *in vitro*, even though they had all the surface receptor markings of proper function and responded to injury in a mouse model. One possible explanation is the lower ploidy of these MKs. Thus, one strategy could be culturing methods that increase the ploidy number and cell size by aggressive maturation. A very simple way to do this is culturing the cells with nicotinamide [62,63], although such treatment has controversial effects on proplatelet robustness and platelet function [64,65]. If increasing the size and ploidy of MKs does increase the number functional platelets, an *ex vivo* strategy for this purpose will likely require the identification of a new factor.

Nevertheless, a unique appeal of immortalized MKs is that they can be cryopreserved, which means they can be stored a much longer period than other derived MKs. This feature could mitigate the low platelet yield per MK, as it might be possible to store and access a larger number of MKs when required. This feature would have even more impact as the number of platelets generated per single immortalized MK is increased (Fig. 1).

One caveat to cell reprogramming is the risk of tumors due to the viral transduction of the transcription factors. Platelets, being anucleate, can be irradiated to kill off any contaminating cells. However, it is still preferred to minimize this potential in every step of the differentiation protocol, which is why alternative reprogramming methods, such as transduction by small molecules are being explored [66]. Feng *et al.* [43] reported that the small molecule iBET151 could be used to inhibit c-Myc expression and still managed to generate immortalized MKs that shed functional platelets. Furthermore, they could improve the yield of $CD41a^+CD42b^+$ cells by mild hyperthermia, which has been observed to increase MK differentiation [67]. In addition, they were able to increase the percentage of $CD41a^+CD42b^+$ MKs by adding

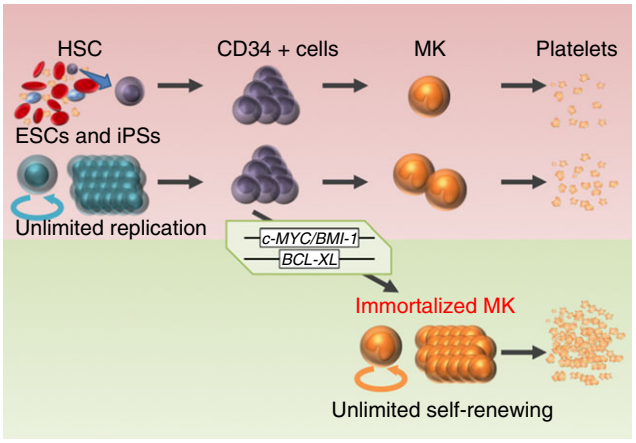

Fig. 1. *Ex vivo* platelet generation has two major obstacles: the expansion of $CD34^+$ cells into megakaryocytes (MKs) and the shedding of platelets from MKs. Prior to embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), hematopoietic stem cells (HSCs) were the only source of $CD34^+$ cells. However, their expansion remains too low for clinical use. Regulating the activation of three transgenes, c-MYC, BMI-1, and BCL-XL, in iPSCs has led to the creation of immortalized MKs. These cells can be cryopreserved and have unlimited replication potential, which offers a solution to the first obstacle.

GM6001 or MMP8-I to the culture. These molecules inhibit metalloproteinases such as ADAM17, which are more active in older populations and shed key receptors from the MK surface to compromise platelet response to injury [68,69]. Yet despite these improvements, the platelet yield remains penury. Schlinker *et al.* [70] proposed a device that could separate platelets from immature MKs, with the latter then recycled in maturation medium to increase their ploidy and platelet yield. Another consideration has the MKs, not platelets, infused and shed platelets in the blood stream resulting in *in vivo* generation [71].

Recapitulating the microenvironment

The potential of immortal MKs may be enhanced with better recapitulation of the natural microenvironment in which platelets are shed. One very simple adjustment is switching the culture from two dimensions to three dimensions, which has been reported to increase the number of progenitors [72]. Furthermore, three dimensions make for more surface area, which could permit more proplatelets to engage with the endothelial wall and therefore enhance the number of platelets acquired. Sullenbarger *et al.* [73] prepared a 3D perfusion bioreactor using scaffolds made of polyester fabric or hydrogel. They showed that coating the scaffold with TPO or fibronectin increased the number of platelets. The platelets, however, were heterogeneous, with a number showing abnormal shape and size. Building on this system, Pallotta *et al.* [74] designed a bioreactor that included both the osteoblast and perivascular niches between which MKs could

migrate by coating silk microtubes with the appropriate extracellular matrix proteins and growth factors. An important advantage of this system was the incorporation of fluorescence imaging to observe MK migration and platelet shedding live. The number of platelets per MK was 200, which is within a magnitude of blood, but only 7% of MKs extended proplatelets toward the pseudo-endothelial wall, suggesting ways that bias the proplatelets to the surface would be advantageous.

The osteoblast niche is a relatively hypoxic environment, a condition that is thought to preserve the pluripotent state and regulate hematopoiesis [75,76]. Therefore, bioreactors that are slightly hypoxic could help expand $CD34^+$ cells. Switching the microenvironment from 5% oxygen at early-stage culturing to 20% at late stage was shown to promote residence in the osteoblast niche and platelet generation [77]. One theory for the stability is that hypoxia causes less expression of vascular cell adhesion molecule 1 and thus denies transition to the perivascular niche [78]. The osteoblast niche can also be stabilized using certain alkenes that enhance the expansion of $CD34^+$ cells [79].

Shear stress is an important factor in platelet number. Thon *et al*. [80] have built a microfluidic bioreactor that along with considering bone marrow stiffness, the extracellular matrix composition, and other factors also incorporates shear stress. They found that the inclusion of shear stress resulted in much faster rates of MK activation, as proplatelets began emerging within seconds of trapping compared with the several hours seen in static conditions and grew at much faster rates, reaching velocities that were comparable with those observed in living mice. The shear stress in their system was generated by parallel flows running around the scaffolds. Nakagawa *et al*. [81] found, however, that a confluent system may be more effective, as they reported that flow intersecting at 60° achieves a 3.6-fold increase from a single-flow system. The reasons for this angle are unclear, but incorporating such a feature into future bioreactors should not be a major challenge.

The impact of flow may be further amplified by extracellular matrix proteins. Results from a mouse model suggest that vWF enhances the evisceration of proplatelets by blood flow [33]. Additionally, shear stress increases the expression of RUNX1 in $CD41a^+$ cells, indicating that biomechanical forces can promote hematopoietic development [82]. Consistently, another biomechanical property, elasticity, was found to enhance the expansion of $CD34^+$ cells [83].

Conclusions

A major challenge in the field of platelet research is generating the massive number of platelets needed for viable patient care. Despite advances in *ex vivo* techniques, there are still ways to go before seeing *ex vivo* platelets reach the clinic. Key advances in the field, however, are bringing this goal closer to reality. Bioreactors that better recapitulate the MK environment by including key extracellular matrix proteins and the effects of shear stress have shown that platelet totals can be increased by several factors. Perhaps more important is the invention of immortalized MKs. These cells can be maintained for several months, which reduces the storage concern of platelets. Introducing immortalized MKs into advanced bioreactors may contribute to the significant leap needed in *ex vivo* platelet generation.

Disclosure of Conflict of Interests

K. Eto has submitted patents related to reference 51 and 81.

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